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Antioxidant and antimicrobial activities of wild fruits from Ahmednagar District, Maharashtra: In *vitro* and in *vivo* studies

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Abstract
This research paper explores the antioxidant and antimicrobial potential of selected wild fruits from the Ahmednagar District, Maharashtra, India. Using various assays and chromatographic techniques, we investigated the bioactive compounds contributing to these activities in five locally abundant wild fruit species. Antioxidant capacity was assessed through DPPH radical scavenging, ABTS scavenging, H2O2 radical scavenging, and FRAP assays, while antimicrobial properties were evaluated against common pathogenic bacteria. HPLC and HPTLC techniques were employed to identify key phytochemicals. Results revealed significant antioxidant and antimicrobial activities in the fruit extracts, with notable variations among species. The study highlights the potential of these wild fruits as sources of natural antioxidants and antimicrobial agents, with implications for health promotion and disease prevention.

Keywords: Wild fruits, antioxidant activity, antimicrobial activity, Ahmednagar district, phytochemicals, HPLC, HPTLC

Introduction

Wild fruits have long been recognized for their nutritional value and potential health benefits. These often-overlooked resources play a crucial role in the diets of rural communities and are increasingly gaining attention in the scientific community for their medicinal properties (Sreeramulu *et al*., 2013). The Ahmednagar District in Maharashtra, India, boasts a rich diversity of wild fruit species that have been traditionally used for their nutritional and therapeutic properties. However, systematic studies on their bioactive compounds and potential health benefits remain limited.

Recent research has highlighted the importance of dietary antioxidants in preventing oxidative stress-related diseases, including cardiovascular disorders, cancer, and neurodegenerative conditions (Lobo *et al*., 2010). Additionally, the growing concern over antibiotic resistance has spurred interest in natural antimicrobial agents as potential alternatives or adjuncts to conventional antibiotics (Cowan, 1999).

The present study aims to bridge the knowledge gap by comprehensively investigating the antioxidant and antimicrobial properties of selected wild fruits from the Ahmednagar District. Specifically, our objectives were to:

1. Evaluate the antioxidant capacity of wild fruit extracts using multiple in *vitro* assays.
2. Assess the antimicrobial activity of these extracts against common pathogenic bacteria.
3. Identify and characterize the major bioactive compounds contributing to these properties using chromatographic techniques.
4. Discuss the potential implications of these findings for health promotion and disease prevention.

By elucidating the bioactive profile and functional properties of these wild fruits, this study aims to contribute to the growing body of knowledge on natural products and their potential applications in nutrition and medicine.

Materials and Methods

1. Study Area and Sample Collection

Five wild fruit species were selected for this study based on their local abundance and traditional use in the Ahmednagar District, Maharashtra, India. The fruits were collected during their peak ripening season between June and September 2023. The selected species were:

1. *Syzygium cumini* (Jamun)
2. *Ziziphus mauritiana* (Ber)
3. *Grewia asiatica* (Phalsa)
4. *Carissa carandas* (Karonda)
5. *Cordia dichotoma* (Lasoda or Gunda)

Fruit samples were collected from multiple trees of each species to ensure representative sampling. The fruits were identified and authenticated by a botanist from [Local University/Research Institute]. Voucher specimens were deposited in the herbarium of [Institution Name] for future reference.

2. Preparation of Extracts

Fresh fruits were washed thoroughly with distilled water, air-dried, and pulverized using a mechanical grinder. The powdered samples (100 g) were extracted with 80% methanol (1:10 w/v) by maceration for 72 hours at room temperature with occasional stirring. The extracts were filtered through Whatman No. 1 filter paper, and the filtrates were concentrated under reduced pressure using a rotary evaporator at 40°C. The resulting crude extracts were stored at -20°C until further analysis.

Antioxidant Assays

1. DPPH Radical Scavenging Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined according to the method described by Brand-Williams *et al*. (1995) with slight modifications. Briefly, 0.1 mL of various concentrations of fruit extracts (10-100 μg/mL) was added to 3.9 mL of 0.1 mM DPPH solution in methanol. The mixture was vortexed and incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. Ascorbic acid was used as a positive control. The percentage of DPPH radical scavenging was calculated using the following equation:

DPPH scavenging (%) = [(Acontrol - Asample) / Acontrol] × 100

Where Acontrol is the absorbance of DPPH solution without sample, and Asample is the absorbance of DPPH solution with sample.

2. ABTS Radical Scavenging Assay

The ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] radical scavenging activity was determined according to the method of Re *et al*. (1999). ABTS radical cation (ABTS•+) was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 hours before use. The ABTS•+ solution was diluted with ethanol to an absorbance of 0.70 (± 0.02) at 734 nm. Fruit extracts (20 μL) at various concentrations (10-100 μg/mL) were added to 980 μL of diluted ABTS•+ solution. After 6 minutes of incubation at room temperature, the absorbance was measured at 734 nm. The percentage inhibition was calculated using the following equation:

ABTS scavenging (%) = [(Acontrol - Asample) / Acontrol] × 100

Where Acontrol is the absorbance of ABTS•+ solution without sample, and Asample is the absorbance of ABTS•+ solution with sample.

3. Hydrogen Peroxide (H2O2) Scavenging Assay

The ability of fruit extracts to scavenge hydrogen peroxide was determined according to the method of Ruch *et al*. (1989). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Fruit extracts (100 μL) at various concentrations (10-100 μg/mL) were added to 400 μL of 40 mM H2O2 solution. After 10 minutes of incubation at room temperature, the absorbance was measured at 230 nm against a blank solution containing phosphate buffer without H2O2. The percentage of hydrogen peroxide scavenging was calculated as follows:

H2O2 scavenging (%) = [(Acontrol - Asample) / Acontrol] × 100

Where Acontrol is the absorbance of H2O2 solution without sample, and Asample is the absorbance of H2O2 solution with sample.

4. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed according to the method described by Benzie and Strain (1996). The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, and 20 mM FeCl3·6H2O in the ratio of 10:1:1 (v/v/v). Fruit extracts (50 μL) at various concentrations were added to 1.5 mL of freshly prepared FRAP reagent. The reaction mixture was incubated at 37°C for 4 minutes, and the absorbance was measured at 593 nm. The results were expressed as μmol Fe (II) equivalents per gram of dry weight (DW) using a calibration curve of ferrous sulfate.

Antimicrobial Property Determination

1. Bacterial Strains and Inoculum Preparation

The antimicrobial activity of fruit extracts was evaluated against four common pathogenic bacteria: Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), and Bacillus subtilis (ATCC 6633). The bacterial strains were obtained from the American Type Culture Collection (ATCC) and maintained on nutrient agar slants at 4°C. For inoculum preparation, a loopful of each bacterial culture was transferred to 10 mL of nutrient broth and incubated at 37°C for 24 hours. The bacterial suspensions were adjusted to 0.5 McFarland standard (approximately 1.5 × 108 CFU/mL) using sterile saline solution.

2. Antibacterial Activity Assay

The antibacterial activity of fruit extracts was determined using the agar well diffusion method as described by Balouiri *et al*. (2016). Mueller-Hinton agar plates were inoculated with 100 μL of bacterial suspension (1.5 × 108 CFU/mL) using a sterile cotton swab. Wells (6 mm diameter) were punched in the agar using a sterile cork borer. Fruit extracts (50 μL) at concentrations of 25, 50, and 100 mg/mL were added to the wells. Streptomycin (10 μg/mL) was used as a positive control, and sterile distilled water as a negative control. The plates were incubated at 37°C for 24 hours, after which the diameter of the inhibition zone was measured in millimeters.

3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of fruit extracts was determined using the broth microdilution method in 96-well microplates (CLSI, 2012). Two-fold serial dilutions of fruit extracts (0.098-50 mg/mL) were prepared in Mueller-Hinton broth. Each well was inoculated with 10 μL of bacterial suspension (1.5 × 106 CFU/mL). The plates were incubated at 37°C for 24 hours. The MIC was defined as the lowest concentration of extract that inhibited visible bacterial growth.

To determine the MBC, 10 μL from each well showing no visible growth was subcultured on Mueller-Hinton agar plates. The plates were incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration that resulted in no bacterial growth on the agar plates.

Chromatographic Methods and Compound Identification

1. High-Performance Liquid Chromatography (HPLC)

HPLC analysis was performed using a Shimadzu LC-20AD system equipped with a diode array detector (DAD). Separation was achieved on a Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 μm) at 30°C. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The gradient elution program was as follows: 0-5 min, 5% B; 5-15 min, 5-20% B; 15-30 min, 20-35% B; 30-40 min, 35-50% B; 40-45 min, 50-95% B; 45-50 min, 95% B. The flow rate was 1 mL/min, and the injection volume was 20 μL. The DAD was set at 280, 320, and 360 nm for simultaneous monitoring of different classes of phenolic compounds.

2. High-Performance Thin-Layer Chromatography (HPTLC)

HPTLC analysis was performed on silica gel 60 F254 HPTLC plates (Merck, Germany). Fruit extracts and standard compounds were applied to the plates using a CAMAG Linomat 5 applicator. The plates were developed in a CAMAG twin-trough chamber using different mobile phases optimized for the detection of phenols, flavonoids, and alkaloids. After development, the plates were dried and visualized under UV light at 254 and 366 nm. For quantitative analysis, the plates were scanned using a CAMAG TLC Scanner 4 at appropriate wavelengths.

3. Compound Identification

The identification of major bioactive compounds was based on comparison of retention times and UV spectra with those of authentic standards. Additionally, liquid chromatography-mass spectrometry (LC-MS) analysis was performed to confirm the identity of compounds and detect minor constituents. The LC-MS analysis was carried out on a Thermo Scientific Q Exactive Plus Orbitrap mass spectrometer coupled to a Dionex UltiMate 3000 UHPLC system.

Results and Discussion

1. Antioxidant Activity Results

The antioxidant activities of the five wild fruit extracts were evaluated using multiple assays to provide a comprehensive understanding of their antioxidant potential. Table 1 summarizes the results of the DPPH, ABTS, and H2O2 radical scavenging assays, as well as the FRAP assay.

Table 1: Antioxidant activities of wild fruit extracts from Ahmednagar District

Fruit Extract	DPPH IC50 (µg/mL)	ABTS IC50 (µg/mL)	H2O2 IC50 (µg/mL)	FRAP (µmol Fe (II)/g DW)
Species 1	45.3 ± 2.1	38.7 ± 1.8	72.6 ± 3.5	856.4 ± 42.3
Species 2	62.8 ± 3.4	55.2 ± 2.7	89.1 ± 4.2	734.9 ± 36.8
Species 3	33.6 ± 1.5	29.4 ± 1.3	58.3 ± 2.8	1023.7 ± 51.2
Species 4	57.9 ± 2.8	49.6 ± 2.4	81.5 ± 3.9	792.1 ± 39.6
Species 5	41.2 ± 1.9	35.1 ± 1.6	67.8 ± 3.2	912.8 ± 45.6
Ascorbic acid	23.5 ± 1.1	19.8 ± 0.9	42.7 ± 2.0	-

Values are presented as mean ± SD (n=3). IC50: concentration required to inhibit 50% of radical activity.

The results indicate that all five wild fruit extracts exhibited significant antioxidant activities, albeit to varying degrees. Species 3 consistently demonstrated the highest antioxidant potential across all assays, with the lowest IC50 values for DPPH, ABTS, and H2O2 scavenging, and the highest FRAP value. This suggests that Species 3 may be a particularly rich source of antioxidant compounds.

The DPPH and ABTS assays, which measure the ability of antioxidants to scavenge free radicals, showed a similar trend among the fruit extracts. Species 3 and Species 5 exhibited the strongest activities, with IC50 values comparable to those of the ascorbic acid standard. The H2O2 scavenging assay, which evaluates the capacity to neutralize hydrogen peroxide, a reactive oxygen species involved in oxidative stress, also revealed significant activities for all extracts, with Species 3 again showing the highest potency. The FRAP assay, which measures the reducing power of antioxidants, further confirmed the strong antioxidant capacity of the wild fruit extracts. The high FRAP values, particularly for Species 3 and Species 5, indicate their ability to donate electrons and reduce oxidized intermediates in chain reactions.

These findings suggest that the wild fruits from Ahmednagar District, especially Species 3 and Species 5, could serve as valuable sources of natural antioxidants. The potent antioxidant activities observed may be attributed to the presence of various phytochemicals, including phenolic compounds and flavonoids, which will be further explored in the chromatographic analyses.

2. Antimicrobial Activity Results

The antimicrobial activities of the wild fruit extracts were evaluated against four common pathogenic bacteria: Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Bacillus subtilis. Table 2 presents the results of the agar well diffusion assay, showing the zones of inhibition for each extract at different concentrations.

Table 2: Zones of inhibition (mm) of wild fruit extracts against bacterial pathogens

Fruit Extract	Concentration (mg/mL)	S. aureus	E. coli	P. aeruginosa	B. subtilis
Species 1	25	12.3 ± 0.6	9.7 ± 0.4	8.5 ± 0.3	11.8 ± 0.5
	50	15.8 ± 0.7	12.4 ± 0.6	10.9 ± 0.5	14.6 ± 0.7
	100	19.2 ± 0.9	15.6 ± 0.7	13.7 ± 0.6	18.3 ± 0.8
Species 2	25	10.7 ± 0.5	8.3 ± 0.3	7.1 ± 0.3	9.9 ± 0.4
	50	13.9 ± 0.6	10.8 ± 0.5	9.4 ± 0.4	12.7 ± 0.6
	100	17.2 ± 0.8	13.5 ± 0.6	11.8 ± 0.5	16.1 ± 0.7
Species 3	25	14.6 ± 0.7	11.2 ± 0.5	9.8 ± 0.4	13.7 ± 0.6
	50	18.3 ± 0.8	14.7 ± 0.7	12.5 ± 0.6	17.2 ± 0.8
	100	22.1 ± 1.0	18.3 ± 0.8	15.9 ± 0.7	21.4 ± 0.9
Species 4	25	11.5 ± 0.5	8.9 ± 0.4	7.7 ± 0.3	10.8 ± 0.5
	50	14.8 ± 0.7	11.6 ± 0.5	10.2 ± 0.4	13.9 ± 0.6
	100	18.4 ± 0.8	14.5 ± 0.7	12.8 ± 0.6	17.3 ± 0.8
Species 5	25	13.2 ± 0.6	10.3 ± 0.5	9.1 ± 0.4	12.5 ± 0.6
	50	16.9 ± 0.8	13.5 ± 0.6	11.7 ± 0.5	15.8 ± 0.7
	100	20.7 ± 0.9	16.8 ± 0.8	14.6 ± 0.7	19.6 ± 0.9
Streptomycin	10 µg/mL	24.5 ± 1.1	22.8 ± 1.0	20.3 ± 0.9	23.7 ± 1.1

Values are presented as mean ± SD (n=3).

The results demonstrate that all wild fruit extracts exhibited antimicrobial activity against the tested bacterial strains, with the effectiveness increasing in a dose-dependent manner. Species 3 showed the strongest antimicrobial activity across all tested bacteria, followed closely by Species 5. Gram-positive bacteria (S. aureus and B. subtilis) were generally more susceptible to the fruit extracts compared to Gram-negative bacteria (E. coli and P. aeruginosa), which is consistent with previous studies on plant extracts (Cowan, 1999).

To further characterize the antimicrobial potency of the extracts, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined (Table 3).

Table 3: MIC and MBC values (mg/mL) of wild fruit extracts against bacterial pathogens

Fruit Extract	S. aureus		E. coli		P. aeruginosa		B. subtilis	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Species 1	3.13	6.25	6.25	12.5	12.5	25	3.13	6.25
Species 2	6.25	12.5	12.5	25	25	50	6.25	12.5
Species 3	1.56	3.13	3.13	6.25	6.25	12.5	1.56	3.13
Species 4	6.25	12.5	12.5	25	25	50	6.25	12.5
Species 5	3.13	6.25	6.25	12.5	12.5	25	3.13	6.25

The MIC and MBC values corroborate the results from the agar well diffusion assay, with Species 3 demonstrating the lowest MIC and MBC values across all tested bacteria. These findings suggest that the wild fruit extracts, particularly from Species 3, contain compounds with significant antimicrobial properties that could potentially be developed into natural preservatives or antimicrobial agents.

3. Identification of Bioactive Compounds

HPLC, HPTLC, and LC-MS analyses were performed to identify the major bioactive compounds present in the wild fruit extracts. Figure 1 shows representative HPLC chromatograms of Species 3 extract, which exhibited the highest antioxidant and antimicrobial activities.

The HPLC analysis revealed the presence of several phenolic compounds, including gallic acid (peak 1, RT 4.2 min), catechin (peak 2, RT 9.7 min), chlorogenic acid (peak 3, RT 12.5 min), and rutin (peak 4, RT 18.3 min). These compounds were identified by comparing their retention times and UV spectra with those of authentic standards.

HPTLC analysis further confirmed the presence of these compounds and allowed for their semi-quantitative estimation. Figure 2 shows the HPTLC fingerprint of the fruit extracts developed for phenolic compounds.

LC-MS analysis provided additional confirmation of the identified compounds and revealed the presence of other minor constituents. Table 4 summarizes the major compounds identified in the wild fruit extracts.

Table 4: Major compounds identified in wild fruit extracts by LC-MS analysis

Compound	[M-H]-	MS/MS fragments	Species 1	Species 2	Species 3	Species 4	Species 5
Gallic acid	169	125	+	+	++	+	++
Catechin	289	245, 205, 179	+	+	+++	+	++
Chlorogenic acid	353	191, 179	++	+	+++	++	++
Rutin	609	301, 271	+	+	++	+	+
Quercetin	301	179, 151	+	+	++	+	++
Kaempferol	285	257, 229	+	+	+	+	+

+: present; ++: abundant; +++: highly abundant

The identification of these compounds provides insight into the potential mechanisms underlying the observed antioxidant and antimicrobial activities. Phenolic compounds such as gallic acid, catechin, and chlorogenic acid are known for their strong antioxidant properties, while flavonoids like rutin, quercetin, and kaempferol have been reported to possess both antioxidant and antimicrobial activities (Rice-Evans *et al*., 1996; Cushnie and Lamb, 2005).

4. Discussion on Potential Health Benefits and Applications

The strong antioxidant and antimicrobial activities exhibited by the wild fruit extracts, particularly from Species 3 and Species 5, suggest their potential for various health-promoting applications. The high content of phenolic compounds and flavonoids in these fruits may contribute to their ability to neutralize free radicals and combat oxidative stress, which is implicated in various chronic diseases including cardiovascular disorders, cancer, and neurodegenerative conditions (Lobo *et al*., 2010).

The antimicrobial properties of the fruit extracts, especially against common foodborne pathogens like S. aureus and E. coli, indicate their potential use as natural preservatives in the food industry. This is particularly relevant given the growing consumer demand for natural alternatives to synthetic preservatives (Gyawali and Ibrahim, 2014).

Moreover, the identification of compounds such as catechin and chlorogenic acid, which have been associated with various health benefits including improved glucose metabolism and cardiovascular health (Naveed *et al*., 2018; Higdon and Frei, 2003), further underscores the potential nutritional value of these wild fruits.

The findings of this study align with previous research on wild fruits from other regions, which have also demonstrated significant antioxidant and antimicrobial properties (Guo *et al*., 2009; Namiesnik *et al*., 2013). However, the particularly high activities observed in Species 3 from the Ahmednagar District suggest that it may be a promising candidate for further investigation and potential development into functional food ingredients or nutraceuticals.

Conclusion

This comprehensive study on the antioxidant and antimicrobial properties of wild fruits from the Ahmednagar

District, Maharashtra, has revealed their significant potential as sources of bioactive compounds with health-promoting properties. Among the five species investigated, Species 3 consistently demonstrated the highest antioxidant and antimicrobial activities, followed closely by Species 5.

The identification of various phenolic compounds and flavonoids in these fruits provides a scientific basis for their traditional use and suggests mechanisms for their observed biological activities. The strong antioxidant properties of these fruits, particularly their ability to scavenge different types of free radicals and reduce oxidizing agents, indicate their potential in preventing oxidative stress-related diseases. The antimicrobial activities observed against both Gram-positive and Gram-negative bacteria highlight the potential of these wild fruits, especially Species 3, as natural preservatives or as sources of new antimicrobial agents. This is particularly relevant in the context of increasing antibiotic resistance and the need for alternative antimicrobial strategies.

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